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SUBMISSION OF A NEW PATENT APPLICATION

COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C.

Sir: Transmitted herewith for filing is the patent application of inventors: Frederick Cassels, Andrew Lees and Richard Schuman

For: MONOCLONAL ANTIBODY WHICH AGGLUTINATES E. COLI HAVING THE CS4-CFA/I FAMILY PROTEIN

Enclosed are
10 pages of specification
1 page of abstract
A Declaration and Power of Attorney

Filing fee has been calculated as shown below:

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Attached hereto is a check in the amount of **\$1010.00**. If further fees are payable, the commissioner is authorized to debit Deposit Account **08-1652** in the amount required to fully pay all fees.

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COMBINED DECLARATION AND POWER OF ATTORNEY

As below named inventor(s) we hereby declare that:

We believe we are the original, first and joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled: "MONOCLONAL ANTIBODY WHICH AGGREGATES E. COLI HAVING THE CS4-CRA/I FAMILY PROTEIN", the specification of which:

Is attached hereto

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 CFR 1.56(a).

We hereby appoint the following attorneys and agents, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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MONOCLONAL ANTIBODY WHICH AGGLUTINATES E. COLI HAVING THE
CS4-CFA/I FAMILY PROTEIN

Field of the Invention:

5 This invention relates to a monoclonal antibody to a consensus peptide of the formula:

VEKNITVTASVDPTIDLLQADGSALPSAVALTYSSPA .

The monoclonal antibody of the invention binds exclusively to the sequence SAVALTYS.

10 Background of the invention:

The effect of E. coli in mammals is dependent on the particular strain of organism. Many beneficial E. coli are present in the intestines. Since the initial association of E. coli with diarrheal illness, five categories of diarrheagenic E. coli have been identified and are presently recognized: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroaggregative (EAggEC), and enteroinvasive (EIEC). These categories are grouped according to characteristic virulence properties, such as elaboration of toxins and colonization factors and/or by specific types of interactions with intestinal epithelial cells. ETEC are the most common of the diarrheagenic E. coli and pose the greatest risk to travelers. E. coli of the family CS4-CFA/I are some of the more common enterotoxigenic E. coli. There is need for vaccines which are specific against this class of E. coli that give rise to antibodies that cross-react with and cross-protect against the more common members of the CS4-CFA/I family. Six members of this family of ETEC fimbrial proteins are CFA/I, CS1, CS2, CS4, CS17 and PCF 0166. ETEC are responsible for high infant mortality in developing countries, with an estimate that almost 800,000 deaths per year due to these organisms. These organisms also cause illness in adult travelers to regions where the disease is endemic.

Colonization factor antigens (CFA) of ETEC are important in the initial step of colonization and adherence of the bacterium to intestinal epithelia. In epidemiological studies of adults and children with diarrhea, CFA/I is found in a large

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percentage of morbidity attributed to ETEC. The CFA/I is present on the surfaces of bacteria in the form of pili (fimbriae), which are rigid, 7 nm diameter protein fibers composed of repeating pilin subunits. The CFA/I antigens promote mannose-resistant attachment to human brush borders with an apparent sialic acid sensitivity.

A study of proteins in *E. coli* belonging to the CS4-CFA/I family resulted in the finding that the N-terminal region of the protein maintains a high degree of sequence identity between members of this group. Immunological evidence shows that cross-reaction exists between members of the family CS4-CFA/I.

Cassels, et al. have identified a consensus peptide of 36 amino acids which acts as an immunogen raising antibodies against the proteins of all members of the *E. coli* family CS4-CFA/I . The region of the protein represented in the subunit encompasses known linear B- and T-cell epitopes of CFA/I. The consensus peptide has a high level of homology to strains bearing six different colonization factors. The consensus peptide is of the formula:

VEKNITVTASVDPTIDLLQADGSALPSAVALTYSPA

Description of the Invention:

It is the purpose of this invention to identify a monoclonal antibody raised to the consensus peptide of Cassels and which will agglutinate all bacteria bearing CS4-CFA/I family proteins.

Preparation of the Immunogen:

A: Iodoacetylation of tetanus toxoid:

To 0.64 ml of a composition containing 18.9 mg/ml (12 mg) of tetanus toxoid (TT) (obtained from SmithKline Beecham) was added 5x HEPES buffer (75 μ l of 0.75 M HEPES, 5 mM EDTA, pH 7.3). The TT was labeled with a 40 fold molar excess of N-hydroxysuccinimidyl iodoacetate (32 μ l of 0.1 mM in dimethyl-formamide). After two hours, the protein was desalted on 2 P6 cartridges (BioRad) in series, equilibrated with HEPES buffer (0.15M HEPES, 1mM EDTA, pH 7.3). The void volume fraction was concentrated to about 0.7 ml using a Macrosep 50 device

(Filtron Corp).

B: Reduction of peptide:

About 10 mg of peptide consensus peptide of the formula
CVEKNITVTASVDPTIDLLQADGSALPSAVALTYSVA

5 was solubilized in 1.1 ml HEPES buffer containing 100 μ l acetonitrile and reduced by the addition of solid dithio-threitol to a final concentration of 0.5 M. After 1 hour the peptide was desalted in two parts on a 1 x 50 cm G10 column (Pharmacia), equilibrated with acetate buffer (10 mM sodium acetate, 0.1 M NaCl, 2 mM EDTA and 0.02% sodium azide at pH 5) and run at 1 ml/min. The void volume fractions were pooled. Ellman's reagent (G.L. Ellman, Arch Biochem & Biophys., 82:70 10 (1959)) was used to determine that the peptide was reduced to a thiol.

15 C: Coupling of peptide to Tetanus toxoid:

Six ml of the reduced peptide was added to 0.3 ml of TT labeled with N-hydroxysuccinimidyl iodoacetate and 1 ml 5x HEPES buffer. After overnight incubation at 4°C, the conjugate was concentrated to about 1 ml using a Macrosep 50 device, then desalted into HEPES buffer using P6 cartridges, concentrated again (Macrosep 50), and, finally, filtered through a 0.45 micron Millex HV filter (Millipore). Evaluation of the protein content using the BioRad assay showed total protein content to be about 2.6 mg/ml.

20 Monoclonal Antibody Production:

A: Preparation of anticonsensus peptide monoclonal antibody:

25 Six BALB/c mice identified as numbers 8378-8383, were immunized with the consensus peptide-TT conjugate. On designated day 1, each mouse was injected subcutaneously with 25 μ g conjugate in 0.2 ml emulsified in 60% complete Freund's adjuvant. On day 23, a serum sample was obtained from each mouse. On day 35, all mice except #8382 received a boost of 10 μ g consensus peptide conjugate in 0.2 ml 60% incomplete Freund's adjuvant. Mouse 8382 was given 10 μ g conjugate of the peptide in 0.1 ml phosphate-buffered saline (PBS).

30 On day 37, mouse 8382 was used for fusion (96-104). This

fusion did not result in production of a monoclonal anti-consensus peptide.

On day 82, the mice received booster immunizations of 10 µg consensus peptide conjugate in 0.2 ml emulsified in 60% incomplete Freund's adjuvant.

On day 85, the spleen from mouse # 8383 was fused with FOX-NY myeloma wherein the myeloma population viability was 97.4%. 1.36×10^8 spleen cells were fused with 1.37×10^7 myeloma cells, using PEG (1400 molecular weight) as a fusogen.

The hybridomas were assigned culture number 96-109.

Hybridomas were planted into 8 96-well tissue culture dishes with 100 µl/well in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10% Hybridoma Serum Free Media (SFM), 100 µM hypoxanthine and 16 µM thymidine (the hypoxanthine and thymidine combination being referred to herein as HT). Eight wells were also planted with FOX-NY myeloma cells only (no hybridomas) as a control. All samples were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 24 hours, all wells received 100 µl RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10% hybridoma SFM, 200 µM hypoxanthine, 0.8 µM aminopterin and 32 µM thymidine. (The hypoxanthine, aminopterin and thymidine combination being referred to herein as HAT.)

Approximately 96 hours after the fusion, the FOX-NY myelomas in control wells appeared to be dead. Many other wells contained growing colonies of hybridomas seven days after fusion. The growing cells were fed by addition of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10% hybridoma SFM and HT. Four days thereafter, the supernatants were tested for the presence of anti-consensus peptide antibodies.

For analysis of peptide binding, an ELISA was used. Nunc Maxisorp stripwells were coated overnight at room temperature with 100 µl/well of consensus peptide at 1 µg/ml PBS. The wells were then washed four times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound material. Each well then received 50 µl of PBS-T. Fifty µl of supernatant was then

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transferred from the cell culture plate to the corresponding wells of the immunoassay dish. Prior to the transfer of the cell culture, wells were screened microscopically to identify wells without hybridomas. One such well from each plate was used as a background control by substituting PBS-T or medium for the culture supernatant. The plates were then sealed and incubated for 30 to 60 minutes at room temperature in the dark in a draft-free environment. The wells were thereafter washed four times with PBS-T to remove unbound material. Each well then received 95 μ l of sheep anti-mouse IgG-HRP (horse radish peroxidase), diluted 1:10000 in PBS-T. Following a 30 minute incubation, the wells were again washed and each well received 100 μ l of tetramethylbenzidine (TMB) substrate solution. The plates were then incubated in the dark for 15 minutes at room temperature, after which the reactions were stopped by addition of 80 μ l of TMB Stop Solution. The absorbance of each well was determined at 450 nm using a Molecular Devices microplate reader.

Absorbance values for 32 of the supernatants from wells with growing hybridomas was greater than 0.200 units. Of these, only two wells, designated CA8 (1.743) and FE8 (1.092) had absorbance values of greater than 1.000. All thirty-two cultures were expanded by transfer into 24-well culture dishes and grown on RPMI 1640 with 10% FBS. Upon retest, only colony FE8 continued to produce antibodies reactive with the consensus peptide. This culture was expanded to growth in a T75 culture flask and samples were cryopreserved.

The isotype of the antibody secreted by 96-109FE8 was determined using a Zymed isotype kit. The results indicated that the antibody was an IgM with a kappa light chain. The 96-109FE8 culture was cloned into 96-well culture dishes by diluting the cells to a concentration of 4.5-5 cells/ml in RPMI 1640 with 20% FBS and 10% hybridoma SFM. Each well received 200 μ l of the cell suspension. Each well was checked for the presence of a single focus of growing hybridomas. The supernatants from each such well were tested for binding of the antibody to the consensus peptide epitope. All of the super-

natants were active, suggesting that all of the surviving cells in the original culture were secretors of the antibody of interest, and that the genotype was stable. One clone, designated 96-109FE8 IH11, was expanded, cryopreserved and used in the production of ascites.

Testing of hybridoma tissue culture supernatant for agglutinating activity:

Bacterial culture: ETEC strains bearing the colonization factors CFA/I, CS1, CS2 and CS4 were grown overnight at 37°C on colonization factor antigen agar [10 gm Casamino acids, 1% (Difco Laboratories, Detroit, MI); 1.5 gm yeast extract (Difco), 0.15%; 0.1 gm MgSO₄·7H₂O), 0.005% (Sigma, St. Louis); 0.008 gm MnCl₂, 0.0005% MnCl₂ (Sigma); 20 gm agar (Difco); q.s. to 1 liter with deionized water]. Those ETEC strains bearing the colonization factors CS17 and PCF 0166 are also grown on colonization factor antigen agar, which was also supplemented with 0.15% bile salts (bile salts #3, Difco). Bacteria were harvested into phosphate buffer saline (PBS) solution and the concentration of bacterial suspension was adjusted to an optical density of 20 (when diluted 1/20 gives an OD of 1.00+/- 0.005 at 600 nm). Bacterial culture supernatant was tested at full strength or serially diluted 1:2 with PBS.

The following assay was used: Eight μ l of bacterial suspension was mixed with an equal volume of tissue culture supernatant dilution on a glass microscope slide (25 x 75 mm) at room temperature. In a separate place on the same slide there is a control consisting of bacterial suspension with 8 μ l of PBS (autoagglutination control). The mixture is rocked back and forth continuously and the agglutination is observed at 10 seconds, 30 seconds, 1 minute and 2 minutes. The results are visually scored as follows:

4 = agglutination in less than 10 seconds with large clumps

3 = agglutination in less than 30 seconds with large clumps

2 = agglutination in less than 60 seconds with medium

clumps

- 1 = agglutination in less than 2 minutes with small clumps
0 = no agglutination within 2 minutes.

Results:

At undiluted tissue culture supernatant (estimated at 1 µg/ml of antibody), no bacterial strains were agglutinated. After concentration of tissue culture supernatant to 20 fold concentration (YM 100 centrifugal ultrafilter, Amicon, Danvers, Massachusetts), only the bacterial strain expressing CFA/I was agglutinated (H10407NM). The monoclonal antibody supernatant was then concentrated 130 fold from original strength and tested. Under these circumstances, the antibody agglutinated all bacteria bearing CS4-CFA/I family proteins.

The hybridoma identified as 96-109FE8 IH11 has been deposited in the American Type Culture Collection in Rockville, Maryland and given the designation ATCC HB-12163.

As indicated above the antibody may be used for purposes of identifying E. coli bearing the CS4-CFA/I protein family. The samples suspected of containing E. coli of the CS4-CFA/I protein may be grown by usual methods in the clinical laboratory. The colonies of organisms may then be suspended by the method disclosed above. The suspended organisms are then exposed to a composition containing at least 30 µg/ml of antibody. In a preferred embodiment, the suspended organisms would be exposed to a composition containing an antibody concentration of 100 to 130 µg/ml. Appropriate samples would include stools from patients suffering from diarrhea and for testing food and environmental samples for contamination with ETEC E. coli organisms.

The monoclonal antibody (MAB) is useful for identifying members of the CS4-CFA/I family in cultures. Assay kits containing the MAB may be prepared and may contain, in addition to the MAB of the invention, agents for tagging for facilitate identification of the MAB/antigen complex. Such tags include radioactive isotopes, fluorescing agents and colorometric indicators. Such agents may be attached to solid supports. For example, an ELISA test kit system may be used

to identify the MAB/antigen complex.

Compositions containing the MAB of the invention may be prepared using as a carrier appropriate for addition to a growth media. Saline and other buffered solutions known in the art are appropriate as carriers for the MAB.

MABs of the invention may also be prepared in pharmaceutically acceptable carrier solutions and may be administered to the infected area to agglutinate the bacteria bearing CS4-CFA/I proteins. Administration would provide means for the compositions to contact the organisms. For example, the compositions could be administered orally in capsules which protect the antibody from destruction in the stomach and duodenum. The compositions are appropriate for use both for short-term prophylaxis and for treatment of ETEC E. coli infections by administration of an ETEC E. coli agglutinating effective amount of the pharmaceutical composition.

What is claimed is:

1. A monoclonal antibody produced by hybridoma 96-109FE8 IH11 which agglutinates bacteria bearing CS4-CFA/I family proteins which binds exclusively and specifically to the sequence SAVALTYS.
2. A monoclonal antibody of claim 1 which is produced by hybridoma 96-109FE8 IH11.
3. A composition of matter comprising a monoclonal antibody of claim 1 in a carrier appropriate for application to a bacteria-containing growth media.
4. A composition of claim 3 wherein the monoclonal antibody has a tag to assist in identification of the antibody/-CS4-CFA/I family protein complex.
5. A composition of claim 4 wherein the tag is a fluorescing agent.
6. A composition of claim 4 wherein the tag is a colorimetric tag.
7. A assay for detecting presence of organisms bearing the CS4-CFA/I family proteins comprising the steps of:
 1. contacting a culture of organisms with a composition of claim 1,
 2. allowing time for the monoclonal antibodies in the composition of claim 1 to interact with the microbial organisms, and
 3. examining the culture after interaction of step 2 to determine whether an CS4-CFA/I family protein/antibody complex has been formed.
8. A composition comprising an antibody of claim 1 in a pharmaceutically acceptable carrier.

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- 11.
- 9. A composition of claim 8 wherein the carrier is saline.
 - 10. A method of treating illness arising from infection with bacteria bearing CS4-CFA/I family proteins by administration of a bacteria-agglutinating effective amount of the composition of claim 8.
 - 11. A method of prophylaxing against illness arising from infection with bacteria bearing CS4-CFA/I family proteins by administration of a bacteria-agglutinating effective amount of the composition of claim 8.

ABSTRACT

A monoclonal antibody to a consensus peptide of the formula:
VEKNITVTASVDPTIDLLQADGSALPSAVALTYSPA .

The monoclonal antibody of the invention binds exclusively to the sequence SAVALTYS and has use as a diagnostic and for prophylaxis against illness arising from *E. coli* which produce the CS4-CFA/I family of proteins and for treatment of disease arising therefrom.